THE CHEMOTHERAPEUTICS OF THE CHAULMOOGRIC ACID SERIES AND OTHER FATTY ACIDS IN LEPROSY AND TUBERCULOSIS

I. BACTERICIDAL ACTION; ACTIVE PRINCIPLE; SPECIFICITY

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Chaulmoogra oil has an empiric reputation in the treatment of leprosy that extends back into antiquity. This reputation is supported by the experience of modern leperologists. The old and less effective method of oral administration of chaulmoogra oil has been replaced, first by subcutaneous and intramuscular injection of oil mixtures, and more recently by intravenous injection of salts of the chaulmoogric acids; and investigators almost unanimously agree that arrest of the disease, improvement, and frequently cure follows adequate treatment with chaulmoogra oil and its derivatives. The importance of the fatty acid therapy of diseases due to the acid-fast group of bacteria has been greatly increased by the report of Rogers[1] on the intravenous use of "sodium morrhuate," the sodium salts of the fatty acids of cod-liver oil, in the treatment of tuberculosis. Rogers maintains that there is nothing absolutely specific in the products of chaulmoogra oil for leprosy, but that the unsaturated fatty acids of both chaulmoogra and cod-liver oils, and by implication the unsaturated fatty acids of any oil, are equally efficacious in either leprosy or tuberculosis. He believes that the unsaturated fatty acids act in some way on the fatty coating of acid-fast bacilli, presumably by injuring the capsule of the bacillus and exposing it to the destructive action of the tissues.

These therapeutic claims for the chaulmoogrates and morrhuates, if substantiated, would be of the greatest importance to medical science and human welfare. Further investigation is necessary, however, not only to confirm these claims, but also to discover the method of action of chaulmoogra and cod-liver oils, to identify and isolate the therapeutically active principle, if such exists, to determine its distribution in

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[1] Brit. Med. Jour., 1919, 1, p. 147; Indian Med. Gaz., 1919, 54, p. 165.

vegetable and animal oils, and to test the specificity of its action in leprosy, tuberculosis and other infectious diseases. Such an investigation conducted in the test tube, where the chemical and bactericidal aspects of the subject can be studied, and in animals in which the experiments can be adequately controlled, will, we believe, supply more precise information on at least some of the problems involved than would clinical observation alone. The scope of the experimental investigation which we have undertaken is indicated in the outline:

1. What is the method of therapeutic action, if any, of chaulmoogra oil in leprosy:
   - A. Direct or bactericidal
   - B. Indirect or physiologic
     - a. Stimulation of nonspecific lipolytic activity of the tissues which might attack the fatty capsule of acid-fast bacilli
     - b. Antigen for the production of more or less specific fat antibodies (Deycke,[2] Kleinschmidt,[3] Lucke,[4] Warden,[5] Young[6])
     - c. Disturbance of the ferment-antiferment balance of the body fluids (Jobling and Petersen[7])
     - d. Production of lymphocytosis, which might act as intermedi aries in the defense of the host
     - e. Nutritional only
   - C. Inactive?
2. What is the active therapeutic principle of chaulmoogra oil:
   - a. Chaulmoogric acid
   - b. Hydnocarpic acid
   - c. Palmitic acid
   - d. Glycerol or other alcohols
   - e. Gynocardin
   - f. Unidentified substance?
3. Is the therapeutic action of chaulmoogra oil specific for:
   - a. Leprosy

   Or does its action extend to:
   - b. Tuberculosis and other infections with acid-fast bacilli
   - c. Infections with nonacid-fast bacteria
4. Is the therapeutically active principle peculiar to:
   - a. Chaulmoogra oil

   Or is it found in:
   - b. Cod-liver oil (Rogers[1])
   - c. Other oils
   - d. Nonfatty substances

DEVELOPMENT OF THE FATTY ACID THERAPY

Chaulmoogra oil was formerly administered as whole oil by mouth in the empirical treatment of leprosy, often accompanied by local applications of the oil to the skin lesions or combined with other treatment. The nauseating

[2] Deutsch. med. Wchnschr., 1907, 33, p. 89.
[3] Berl. klin. Wchnschr., 1910, 47, p. 57.
[4] Jour. Immunol., 1916, 1, p. 456.
[5] Jour. Infect. Dis., 1918, 23, p. 504.
[6] Jour. Path. & Bacteriol., 1919, 22, p. 224.
[7] Jour. Exper. Med., 1914, 19, p. 239.

property of this oil and the long course of treatment necessary to obtain therapeutic results seriously interfered with its efficient use. Nevertheless, improvement and even cure of leprosy attributed to chaulmoogra oil administered by this method have been reported (Dyer,[8] Hopkins,[9] Connel,[10] Hollmann and Dean[11]). The most comprehensive report published on the oral administration of chaulmoogra oil in leprosy is that of Hopkins, who gives the results of its use during fifteen years at the Leper's Home of Louisiana. Excluding cases on which a fair trial of the treatment had not been given, his figures are:

1. Incipient cases	82
Discharged cured	17%
Remaining at the Home, all lesions having disappeared	4%
Remaining at the Home in an improved condition	24%
Absconded in an improved condition	24%
Remaining at the Home, the disease apparently arrested	14%
Worse	8%
Died	4%
2. Advanced cases	88
Remaining at the Home in an improved condition	12%
Remaining at the Home, the disease apparently arrested	5%
Absconded in an improved condition	9%
Little change	28%
Disease became terminal	20%
Died in the terminal stage	23%

Since gastric disturbance and slowness of action are serious obstacles to treatment by oral administration, a number of mixtures of chaulmoogra oil with other substances have been proposed to render it more fluid, improve its absorption and reduce its irritating property, thus making it suitable for subcutaneous and intramuscular injection (Jeanselme,[12] Brocq and Pomaret,[13] Mercado and Heiser,[14] Hollmann and Currie[11]). Of these mixtures, that of Mercado and Heiser,[14] generally known as the Heiser mixture, has been used most extensively. This mixture consists of chaulmoogra oil, 60 c c; camphorated oil, 60 c c, and resorcin, 4 gm.

In 1913 Heiser[14] reported the apparent cure of two cases of leprosy by subcutaneous injections of this chaulmoogra oil mixture, combined with vaccine treatment, and in one case with the oral administration of chaulmoogra oil. Later[15] he reported the apparent cure of two other cases by subcutaneous injections of chaulmoogra oil mixture alone for a period of from 4 to 8 months. In a third communication[16] Heiser gave the results of this method of treatment in 12 additional cases. Some patients were apparently cured, others showed great improvement, and in all the disease had been arrested.

The subcutaneous or intramuscular injection of chaulmoogra oil mixtures has been used subsequently in the treatment of leprosy by Hopkins[9] in 9 cases,

[8] N. Y. Med. News, 1905, 87, p. 199.
[9] New Orleans Med. and Surg. Jour., 1916, 69, p. 223.
[10] Jour. Trop. Med. and Hyg., 1919, 22, p. 37.
[11] Jour. Cutan. Dis., 1919, 37, p. 367.
[12] Presse méd., 1911, 19, p. 989.
[13] Bull. Soc. franç. de dermat. et syph., 1913, 24, p. 70.
[14] U. S. Pub. Health Rept., 1913, 28, p. 1855.
[15] Ibid, 1914, 29, p. 21.
[16] Ibid., p. 2763; Am. Jour. Trop. Dis. and Prev. Med., 1914, 2, p. 300.

by McCoy and Hollmann[17] in 42 cases, by Armellini[18] in 1 case, by Bercovitz[19] in 14 cases, by Coghill[20] in 7 cases, by Hall[21] in 90 cases, by Cadbury[22] in 26 cases, by Connel,[10] Hollmann and Dean[11] and others, with encouraging results. The last named authors report 12 leper patients in Hawaii who became bacteriologically negative after treatment by intramuscular injections of chaulmoogra oil mixtures, of which only 2 subsequently had a recurrence, one within 7 months and the other within 2 years.

Recently Hollmann and Dean[11] have prepared and used by intramuscular injection the ethyl esters of fractions of the fatty acids of chaulmoogra oil in the treatment of leprosy in Hawaii. These acids, after isolation by the ordinary chemical methods, were separated into fractions by fractional crystallization and the fractions converted into their ethyl esters. The four fractions used in the experiments were:

A. Ethyl ester of chaulmoogric acid.

B. Ethyl ester of acids crystallizing from alcohol with chaulmoogric acid in the initial separation.

C. Ethyl ester of acids soluble in 92% alcohol in the first separation and which form soluble lead salts.

D. Ethyl esters of acids forming lead salts insoluble in ether.

These authors report 26 cases treated 4 months or longer by these several fractions. Patients receiving fractions C and D have shown the greatest improvement. Of these 26 cases, all have shown improvement—many, marked improvement. Eight have already become bacteriologically negative and have been paroled from segregation.

In this connection it is interesting to note that Brill and Williams[23] state that the ethyl esters of chaulmoogric and hydnocarpic acids, sometimes known as antileprol, were found to be ineffective on leprosy in the Philippine Islands.

The next advance in the chaulmoogra oil therapy of leprosy was the attempt to devise preparations suitable for intravenous injection. Vahram,[24] in order to avoid gastric disturbances resulting from the use of chaulmoogra oil by stomach and the pain and abscess formation incident to subcutaneous injection, prepared an emulsion of chaulmoogra oil for intravenous injection. The formula for this "pseudo-solution," as given by him is: gum acacia, 0.144 gm.; chaulmoogra oil, 0.00072 gm. The oil is added to the gum acacia. After desiccation cold, the mixture is submitted to long porphyrization, then put in suspension in the initial volume of liquid and sterilized at 110 C. The emulsion prepared in this manner is characterized by the minuteness of the suspended globules of oil, which are said to approach the dimensions of colloidal granules. Vahram thinks this property not only renders the emulsion suitable for intravenous injection but that it, in consequence of the great multiplication of surface, should produce an intensification of the therapeutic action. Stévenel,[25] in collaboration with Noc, prepared an emulsion for intravenous injection by shaking chaulmoogra oil with N/1 Na_2CO_3. Oil globules of emulsions prepared by this method are said to be as small or smaller than red blood corpuscles.

[17] U. S. Pub. Health Bull., 1916, No. 75, p. 3.
[18] Clin. Dermosifilopat. d. r. Univ. di Roma, 1917, 35, p. 103.
[19] Jour. Am. Med. Assn., 1917, 68, p. 1960.
[20] Ann. Trop. Med. and Parasit., 1917, 11, p. 205.
[21] Trop. Dis. Bull., 1919, 13, p. 13.
[22] China Med. Jour., 1918, 32, p. 226.
[23] Philippine Jour. Sc., 1916, Sect. A, 11, p. 78; 1917, Sect. A, 12, p. 207.
[24] Progrès med., 1916, 31, p. 19.
[25] Bull. Soc. Path. Exot., 1917, 10, p. 684.

Vahram,[24] Hopkins[9] and Stévenel[25] each report improvement in two cases of leprosy undergoing intravenous treatment with these emulsions; but apparently these patients had not been under treatment long enough to determine the ultimate result.

Rogers[26] appears to be the first to use the soluble salts of the fatty acids of chaulmoogra oil intravenously in leprosy, although such salts and their administration by mouth are mentioned by Roux,[11] Trapezinkoff,[11] Dyer,[8] Desprex,[27] Amaral and Parambos,[28] and Hollmann and Currie.[11] Rogers separated the total fatty acids of chaulmoogra oil by the ordinary chemical methods. These crude fatty acids were then separated into fractions of different melting points by dissolving in hot alcohol and removing the acids that crystallized out at different temperatures as the solution cooled. The first fraction, which constituted about two thirds of the total, had a melting point of from 40.8 to 43 C. and is designated as fraction A; the second, with a melting point of from 37 to 40 C., is fraction B, and the remainder, which was liquid at room temperature (28 C.) in Calcutta, is fraction C. These fractions, which may be purified by dissolving in ether, were converted into water-soluble sodium salts by titrating with sodium hydroxid, using phenolphthalein as an indicator, and are called by Rogers "sodium gynocardates."

Rogers states that the sodium salt of fraction A is only slightly soluble in water, is unsuited for either subcutaneous or intravenous injection, and that it is doubtful whether it has any therapeutic value. He used the sodium salts of fractions B and C combined, first subcutaneously and later intravenously[29] in the treatment of leprosy. Still later Rogers[30] decided that the fraction of the fatty acids of chaulmoogra oil having a higher melting point of from 49 to 62 C. yield sodium salts sufficiently soluble and that they are more potent in the treatment of leprosy than the salts of fractions having lower melting points. After two years' experience with sodium gynocardate in leprosy he found that subcutaneous injections do not produce reactions in leprous tissues and are less effective therapeutically than intravenous injections. He employed the intravenous route very extensively, having given over 1,000 intravenous injections of the drug, without any ill effects beyond temporary giddiness and headache and occasional localized clotting in the veins, while the results have been most encouraging. All of the patients have shown improvement. The lesions have disappeared and become bacteriologically negative in 50% of the cases treated within 3 years of the onset of the disease, including cases treated for only from 3 to 12 months; while in cases of from 3 to 15 years' duration, 25% have cleared up under treatment.

Rogers' sodium gynocardate has been used subcutaneously and intravenously by Cadbury,[22] by Carthew[31] in 13 cases, by Muir[32] in 30 cases, by Peacock[33] in 6 cases, by Rogers in 36 cases, by Connel,[10] Muir,[34] in part combined with sodium morrhuate, in 23 cases of leprosy with equally encouraging results.

[26] Lancet, 1916, 1, p. 288.
[27] Lepra, 1900, 6, p. 218.
[28] Bull. gén. de therap., 1908, 155, 415; Lepra, 8, p. 249.
[29] Brit. Med. Jour., 1916, 2, p. 550.
[30] Ind. Jour. Med. Research, 1917, 5, p. 227.
[31] Ind. Med. Gaz., 1918, 53, p. 407.
[32] Ibid., p. 209.
[33] Ibid., p. 95.
[34] Ibid., 1919, 54, p. 130.

Spittle[35] alone reports unfavorably, stating that the reaction was always more or less severe even after small doses and in no case, even after several months, did benefit result.

Recently Rogers[36] reported the results of experiments with the sodium salts of the fatty acids of the oil from the seeds of Hydnocarpus wightiana, a plant closely related to Taraktogenos (Hydnocarpus) kurzii from which chaulmoogra oil is obtained. This oil is said to contain a larger proportion of hydnocarpic acid than chaulmoogra oil. The results of the treatment with these salts, which he now designates as "sodium hydnocarpate," Rogers considers very satisfactory. There was a great reduction and frequent total disappearance of the lepra bacilli, and the number of cases in which the lesions disappeared in less than a year is considered noteworthy. In only one of fourteen cases was the improvement slight.

The promising results obtained in the treatment of leprosy with chaulmoogra oil and its products would naturally suggest their trial in tuberculosis; yet there have been surprisingly few attempts to apply chaulmoogra oil therapy to this disease. Hernandez[39] gives an account of a few experiments, of which only a review has so far been available. He found that the addition of 2% of chaulmoogra oil to the culture medium inhibited the growth of B. tuberculosis, and that treatment of experimentally infected guinea-pigs seemed to confirm the destructive action of chaulmoogra oil on tubercle bacilli. Six patients suffering with tuberculosis were treated with small injections (subcutaneous?) of chaulmoogra oil, 1-2 c c at from 20 to 30 day intervals; all symptoms are said to have subsided in some of the patients.

Rogers[37] suggested the use of sodium gynocardate (chaulmoograte) in the treatment of tuberculosis in 1916; but, since in rare cases of leprosy prolonged febrile reaction and temporary exacerbation of the disease may follow intravenous administration of sodium gynocardate, he hesitated to use it in tuberculosis. For this reason he was led to try sodium morrhuate, the sodium salts of the fatty acids of cod-liver oil, in the treatment of tuberculosis. Without preliminary cultural or animal tests, Rogers has used this salt in the treatment of human tuberculosis, and has supplied it to several clinicians in India for trial, and sodium morrhuate has been put on the market as a specific treatment for tuberculosis.

Rogers[1] states that intravenous injections of sodium morrhuate produce a slight febrile and local congestive reaction, similar to that produced by sodium gynocardate in leprosy, which clearly points to a definite action on the tuberculous tissue. Improvement in phthisical cases is seen in the reduction and cessation of the fever, diminution of the expectoration and cough, and steady gain in weight. In addition, the tubercle bacilli in the sputum gradually decrease in number, and may in time disappear. Moreover, they commonly show deficient acid-fast staining and a granular or beaded appearance, indicating that they are actually being destroyed within the tissues. Furthermore, Rogers says that a year's experience has shown that sodium morrhuate is of great value in leprosy. He believes that the unsaturated fatty acids of both chaulmoogra and cod-liver oils act in some way on the coating of acid-fast bacilli, that of the tubercle bacillus having been shown to contain palmitic and other unsaturated fatty acids.

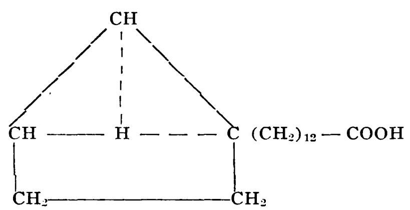
[35] Ibid., 1918, 53, p. 33.
[36] Brit. Med. Jour., 1919, 1, p. 147.
[37] Jour. Am. Med. Assn., 1918, 71, p. 1177.

The Chemistry of Chaulmoogra Oil

Chaulmoogra oil is a fixed oil, expressed cold from the seeds of Taraktogenos (Hydnocarpus) kurzii King, a tree native of Burma. The most complete and trustworthy investigation of the chemistry of chaulmoogra oil is that by Power and collaborators (1904-1907).[38] Chaulmoogra oil has a melting point of from 22 to 23 C., a specific gravity of 0.951 at 25 C., an acid value of 23.9, a saponification value of 213.0 and an iodin value of 103.2. It is optically active, (a) $\frac{D}{150} = +52.0$ C. Like other fixed oils and fats, chaulmoogra oil consists essentially of glyceryl triesters of fatty acids. Of chief interest is the fact that Power and his collaborators have isolated from chaulmoogra oil and studied chemically a series of unsaturated fatty acids which have a structure and properties entirely different from all other known fatty acids. Although these fatty acids have the same empiric formula as the fatty acids of the linoleic series ($C_nH_{2n-4}O_2$) found in linseed and many other vegetable oils, they differ from these and all other fatty acids in their molecular structure. The fatty acids hitherto known are aliphatic or acyclic compounds, which are optically inactive and have their atoms or radicals arranged in an open chain, of which the following structural formula of oleic acid is an example:

$$CH - (CH_2)_7 - CH = CH - (CH_2)_7 - COOH$$

On the other hand, the fatty acids of the chaulmoogric series are optically active and have their atoms arranged in a closed carbon chain or ring; that is, they are cyclic compounds, as is illustrated by the structural formula proposed by Barrowcliff and Power for chaulmoogric acid:



Power and his collaborators isolated two fatty acids of this cyclic series from chaulmoogra oil. One, constituting the larger proportion of the fatty acids, has a melting point of from 68 to 69 C., an empiric formula $C_{18}H_{32}O_2$, and is designated as chaulmoogric acid; a lower isomer, having a melting point of from 59 to 60 C. and an empiric formula of $C_{16}H_{28}O_2$, is designated as hydnocarpic acid. These authors also suggest that chaulmoogra oil may contain other lower isomers of this series of fatty acids. In addition to this series of cyclic fatty acids, chaulmoogra oil contains a small amount of a common, saturated, aliphatic fatty acid, palmitic acid, together with glycerol and phytosterol; and, since the oil is expressed cold from the seeds, it may possibly contain a small amount of the cyanogenic glucosid, gynocardin (Power and Lees) and other nitrogenous substances.

[38] Power and Gornall: Jour. Chem. Soc., 1904, 85, p. 851; Power and Lees: Ibid., 1905, 137, p. 349; Power and Barrowcliff: Ibid., 884; Barrowcliff and Power: Ibid., 1907, 91, p. 557; Power, F. B.: Am. Jour. Pharm., 1915, 87, p. 493.

Chattopadhyay[39] has taken exceptions to the conclusions of Power as to the cyclic structure of the chaulmoogric acid series; but Brill,[23] investigating the oil of Hydnocarpus venenata, a species closely related to Taraktogenos (Hydnocarpus) kurzii, has confirmed the presence of a fatty acid series in this group of plants having a closed carbon chain.

METHODS

The present report is concerned exclusively with a study of the antiseptic and bactericidal actions of chaulmoogra oil and its constituents, the identification and isolation of the bactericidally active substance of chaulmoogra oil, the determination of the specificity of its bactericidal action for acid-fast bacilli, and an investigation of the presence or absence of this bactericidal substance in cod-liver and other oils. The chemotherapeutic investigations on experimentally infected animals will be described separately in the near future.

The methods consist (1) in the separation of chaulmoogra and other oils into fractions and chemical constituents, (2) the preparation of water soluble salts of these fractions and constituents, and (3) tests of the antiseptic and bactericidal activities of these salts against acid-fast and other bacteria.

Since the fixed oils consist essentially of glyceryl triesters of fatty acids, the fundamental analytic procedure has consisted in the separation of the fatty acids from the glycerol and other nonsaponifiable constituents. The ordinary chemical methods of saponifying with alcohol-potash solution and recovering the fatty acids by decomposing the potassium soaps with dilute sulphuric acid were employed. In the case of chaulmoogra oil, the fatty acids, which are solid at room temperature, after being washed free from sulphuric acid, were sometimes purified by dissolving in ether and washing with water and then recovered by evaporating the ether. The fatty acids of cod-liver and linseed oils are fluid at room temperature and float on the surface of the solution as an oily layer; consequently, a slightly different procedure is necessary to recover the separated fatty acids. This was accomplished by dissolving the fatty acids in ether and washing in a separating funnel.

Rogers' fractions of the fatty acids of chaulmoogra oil, used by him in the treatment of leprosy, were prepared by a modification of the method described by this author. He dissolved the total fatty acids in hot 95% alcohol, and removed those that crystallized out at different temperatures as the solution cooled. The essential point is to obtain successive fractions of the total fatty acids crystallizing out of alcohol and having melting points of from 40.8 to 43 C., 37 to 40 C. and below 28 C., respectively. We have found it more practicable to obtain fractions having melting points within the required limits by fractional crystallization of the total fatty acids from cold solutions in three parts of 95% alcohol. Fraction A, with a melting point of 42 C., is a white crystalline solid; fraction B, with a melting point of 37 C., is a slightly yellowish, somewhat amorphous solid; and fraction C is a light brown fluid at 28 C. and a yellowish pasty mass at from 15 to 20 C.

The isolation and purification of the chemically distinct fatty acids of chaulmoogra oil was done by the methods described by Power and Gornall and Power and Barrowcliff.[38] Pure chaulmoogric acid was separated from the total fatty acids by repeated crystallization from 95% alcohol, followed by recrystallization from petroleum ether until a constant melting point of from 68 to 69 C. of the crystals was obtained. This acid, which constitutes the

[39] Am. Jour. Pharm., 1915, 87, p. 473.

greater part of the fatty acids of chaulmoogra oil, crystallizes in colorless glistening leaflets, insoluble in water, but sparingly soluble in and readily crystallized from ordinary organic solvents, with the exception of ether and chloroform in which it is readily soluble cold. Pure hydnocarpic acid was obtained from the noncrystalline residue from chaulmoogric acid by fractional precipitation with barium acetate, recovery of the fatty acids by decomposition of the barium salt fractions with dilute hydrochloric acid, and purification of the fractions last precipitated by crystallization from alcohol and finally from petroleum ether until a constant melting point of from 59 to 60 C. was obtained. This acid, like chaulmoogric acid, crystallizes as colorless, glistening leaflets. The palmitic acid fraction, remaining after the removal of the chaulmoogric and hydnocarpic acids, is a light brown oily fluid at room temperature. It undoubtedly contains small amounts of dissolved chaulmoogric and hydnocarpic acids or lower isomers, but it was not found necessary to purify this fatty acid for use in our experiments.

On account of the insolubility of the oils and their fatty acids in water, it was necessary to convert the fatty acids into water soluble sodium or potassium salts, in order to have solutions suitable for bactericidal tests. Rogers[29, 30] has shown that the therapeutic activity of chaulmoogra oil and its fatty acids is not decreased but rather increased by combining the fatty acids with sodium, due probably to the greater solubility and absorbability of the salts. These soluble salts were prepared by titrating the oil or its fatty acid fractions with half normal sodium or potassium hydroxid, using a suitable indicator. The standard chemical method requires that fatty acids be titrated in hot alcohol with phenolphthalein as an indicator because in aqueous solutions the salts of the fatty acids undergo hydrolytic dissociation. The fatty acid being insoluble is removed from the sphere of chemical action and the sodium ions combining with water interfere with the correct titration. The presence of ethyl alcohol in a concentration of 40% or higher prevents this hydrolytic dissociation and permits a correct titration. In our experience, however, the titration of fatty acids of chaulmoogra oil in 70% alcohol with phenolphthalein as an indicator gives a solution of the sodium or potassium salts which, when diluted with water, is strongly alkaline to litmus, is clear or only slightly clouded, but precipitates on standing and has a low bactericidal activity. On the other hand, when properly titrated in water the solution has a lower titer, is neutral or only feebly alkaline to litmus, is clouded but does not precipitate on standing, and possesses the maximum bactericidal activity. The practical difficulty in titrating in water is that phenolphthalein or any other of a series of indicators tested does not show a sharp end-point, and serves at best only as a rough control of the titration. Consequently, it was necessary to determine experimentally the titer of the chaulmoogrates giving the maximum bactericidal activity. The essential points in the titration are that all of the fatty acids are saponified, that the reaction to litmus is nearly neutral, and that the solution does not precipitate on standing. Since only the sodium and potassium salts of these fatty acids are soluble in water, our tests have necessarily been confined to them.

The correct titer of the oil or its fatty acids having been determined, a 1% solution for testing its antiseptic and bactericidal action was made up as follows: One gram of the oil or fatty acids was accurately weighed and placed in a 100 c c volumetric flask, and the required amount of normal sodium hydroxid and a little distilled water added; the flask and its contents were then heated over a water bath and repeatedly shaken until the fatty acids were

completely saponified. The flask was then filled up to the graduation mark with distilled water and sterilized. This gave a 1% solution, not of the sodium chaulmoograte, but of the oil or fatty acids.

A 1% solution of the oil or fatty acids, instead of a 1% solution of the salts of the fatty acids, was made because we believe that in all comparative bactericidal and chemotherapeutic tests comparison should be made of the bactericidally and chemotherapeutically active atoms or radicals and not of the whole compound containing varying kinds and amounts of bactericidally and chemotherapeutically inert atoms and radicals. The latter serve to render the active atoms or radicals more soluble, absorbable and parasitotrophic, or less irritating and toxic to the host. The sodium in the sodium chaulmoogrates serves only to render the fatty acids soluble and plays no direct part in the bactericidal activity of the compound, as shown by the facts that potassium can replace sodium and that any excess of either base over that necessary to secure solution of the fatty acids, even within the limits of chemical combination with the fatty acids, depresses the bactericidal activity of the solution.

For testing the antiseptic and bactericidal activity of chaulmoogra and other oils and their constituents, cultures of the following acid-fast bacilli were employed: B. leprae muris (Hollmann); B. leprae hominis (Levy); B. smegmatis; B. lymphangitidis bovis (Traum); B. tuberculosis avis; B. tuberculosis bovis and B. tuberculosis hominis. Allied to the acid-fast bacilli are the streptothrices, filamentous branching fungi, often having a bacillary stage and a more or less acid-fast staining reaction, and causing streptothriciases in man and animals. A considerable series of these organisms have been used in our experiments, including one or more strains of the following species: Streptothrix asteroides, S. caprae, S. eppingeri, S. hominis, S. madurae and S. nocardi. For determining the specificity of the bactericidal action of the chaulmoogrates against acid-fast bacilli, cultures of the following nonacid-fast bacteria were used: B. coli, B. typhosus, B. dysenteriae Shiga, B. mucosus, B. pestis, Spirillum cholerae-asiaticae, Staph. aureus and Streptococcus (nonhemolytic). We are indebted to Dr. K. F. Meyer of this laboratory for many of these cultures, and to Dr J. Traum of the Veterinary Division of the Department of Agriculture, University of California, for a culture of Bacillus lymphangitidis bovis, a more or less acid-fast bacillus which he has found in and isolated from a type of chronic lymphangitis in cattle.

Our experiments with the cultures from human and rat leprosy are open to a certain criticism. A considerable series of more or less acid-fast organisms have been cultivated by different investigators from the lesions of human and rat leprosy, none of which have proved to be identical with B. leprae Hansen or the variety of it found in rat leprosy. The most that can be said of our cultures is that they are acid-fast bacilli cultivated by competent bacteriologists from the lesions or blood of human or rat leprosy, and, in the case of the culture from rat leprosy, that the organism was found to be pathogenic for rats, in which it produces a disease similar to rat leprosy. However, the criticism to which these cultures are open is largely, if not wholly, met by the fact that the bactericidal activity of the chaulmoogric acid series has been found to be specific for all members of the acid-fast group of bacteria.

The in vitro method of testing germicidal action of drugs as a guide to their use in chemotherapeutics has fallen into disfavor, because it has been found that germicidal action in the test tube does not always mean germicidal activity in the animal body in dosage within the limits of tolerance of the patient. However, sweeping criticism of this method is unjustified and is based

on a misconception of its purpose and limitations. The in vitro method of testing germicidal action is an analytic method which enables us to exclude the organotrophic reactions of the host and consequently to obtain uncomplicated information on the action of the drug on the parasite. Moreover, and very important for our purpose, the use of this method has enabled us to exclude the possible indirect or physiologic actions of chaulmoogra oil in leprosy and obtain exact data on the bactericidal activity of this oil and its constituents against acid-fast bacteria.

The tests of the antiseptic action of chaulmoogric and other fatty acids were made by adding with a sterile pipet, graduated to hundredths c c, the required amounts of a sterile 1 or 0.1% solution of the salts to a series of tubes or flasks containing definite quantities of suitable fluid culture medium, so that dilutions of 1:1,000 up to the limits of antiseptic action were obtained. These culture tubes or flasks, together with controls of plain medium, were then inoculated with the organism to be tested, the openings sealed to prevent evaporation and incubated at 37.5 C. for at least twice the length of time necessary to obtain the maximum growth in the controls. These cultures were examined from time to time and the amount of growth or absence of growth recorded and, at the termination of the experiment, microscopic examination for purity was made of the cultures showing growth.

Another method was employed to determine the limits of bactericidal action, since in the antiseptic tests growth might be inhibited without the bacteria being killed. Culture tubes containing measured amounts of suitable culture medium were inoculated with the organism to be tested and incubated until a slight but distinct growth had developed; then the proper dilutions of the fatty acid salts were added to the growing cultures, excepting controls, the tubes replaced in the incubator, and at definite intervals transplants of one small loopful were made from each tube to tubes of fresh mediums, which were incubated and the growth results recorded. The amount of the bactericide transferred by this method to the transplant culture gave a dilution in every case far beyond the limits of antiseptic action. This method, instead of the standard method of testing bactericidal action, was used in our work because growth of the several varieties of tubercle bacilli after submergence and drying is uncertain. In the case of B. tuberculosis another method was used in an attempt to control the limits of bactericidal action. To a series of tubes containing 10 c c of a suspension of tubercle bacilli in salt solution definite dilutions of the chaulmoograte were added, two tubes being reserved as controls. After incubation at 37.5 C. for 24 hours, 0.5 c c of the suspension of tubercle bacilli from each tube, well shaken up, was injected subcutaneously into guinea-pigs. The bactericidal action of the different dilutions of chaulmoogrates was determined by the absence or presence of infection at necropsy of the guinea-pigs several months after inoculation.

EXPERIMENTAL DATA

1. ANTISEPTIC AND BACTERICIDAL ACTIONS OF THE TOTAL FATTY ACIDS OF CHAULMOOGRA OIL

Rogers,[31] Hollmann and Dean[11] and others claim superior therapeutic results in leprosy from the use of the salts or esters of the fatty acids of chaulmoogra oil; therefore it seemed probable that the active therapeutic principle of this oil must be contained in the fatty acid fraction. Consequently the antiseptic and bactericidal properties of the total fatty acids of chaulmoogra oil were first investigated. In our preliminary experiments the total fatty acids were titrated in water with N/1 Na_2CO_3, using phenolphthalein as indicator, and

the tests were made on Hollmann's bacillus of rat leprosy cultivated in glycerol veal broth. The results of the first three experiments, in which successively higher dilutions were tested to determine the limits of antiseptic action, and in which transplants were made to determine whether or not the inhibited cultures were actually killed, are combined in table 1.

TABLE 1

PRELIMINARY TESTS OF THE ANTISEPTIC AND BACTERICIDAL ACTIONS OF THE SODIUM SALTS OF THE TOTAL FATTY ACIDS OF CHAULMOOGRA OIL ON B. LEPRAE MURIS

Proportion of Chaulmoogric Acids Added to Culture Medium	Growth in Treated Cultures		Growth in Transplant Cultures
	Macroscopic	Microscopic	
From 1:1,000 to 1:60,000	0	0	0
1:70,000	0	0	+
1:80,000	0	0	0
1:90,000	0	0	0
1:100,000	0	0	0
1:125,000	0	0	+
1:150,000	+	+	+
1:175,000	+	+	+
1:200,000	+	+	++
1:500,000	+	+	+++
1:600,000	++	++	+++
1:700,000	++	++	+++
1:800,000	++	++	+++
1:900,000	++	++	+++
1:1,000,000	++	++	+++
1:1,500,000	+++	+++	+++
1:2,000,000	++++	++++	++++
Controls	++++	++++	++++

From these results it appears that the sodium salts of the total fatty acids of chaulmoogra oil have markedly antiseptic and bactericidal actions on Hollmann's culture of rat leprosy bacillus. Growth of the cultures was totally inhibited up to the dilution of 1:125,000 and partial inhibition, as shown by feeble or delayed growth, extended up at least to the dilution of 1:1,000,000. Complete bactericidal action, as shown by absence of growth in transplants to fresh culture medium, extended up to the dilution of 1:100,000, with the exception of 1:70,000 which showed growth. The tendency of chaulmoogrates to skip antiseptic and bactericidal action is characteristic and will be discussed later.

In the next series of antiseptic tests of the sodium salts of the total fatty acids of chaulmoogra oil, the fatty acids were titrated in 70% alcohol with N/2 NaOH using phenolphthalein as indicator, according to the standard chemical method. The titer of 1 gm. of the fatty acids was 3.8 c c N/1 NaOH. The tests were made, as in the first series, against the bacillus of rat leprosy in glycerol veal broth. The antiseptic action of this solution proved by repeated tests to be only about one-half that of the first solution titrated in water. The cause of this depression of the antiseptic action of the fatty acids titrated in alcohol was obscure. It seemed possible that it might be due (1) to a higher initial acidity of the culture medium, perhaps increased by the growth of the cultures, sufficient to precipitate some of the fatty acids; or (2) to the high titer of the chaulmoograte solution titrated in alcohol. Titration of the culture medium before and after the growth of the cultures in it showed that the reaction was the same in both series of tests and that the growth of the rat leprosy bacillus decreased rather than increased the initial acidity of the medium. Com-

parative experiments were then undertaken to determine the influence of the reaction of the culture medium and of the titer of the chaulmoogratess on the antiseptic action of the sodium chaulmoogrates against B. leprae muris, the results of which are recorded in table 2.

TABLE 2

THE INFLUENCE OF VARIATIONS IN THE TITER OF THE FATTY ACIDS AND IN THE REACTION OF THE CULTURE MEDIUM ON THE ANTISEPTIC ACTION OF THE CHAULMOOGRIC ACIDS ON B. LEPRAE MURIS

Experiment	Titer of the Fatty Acids N/1 NaOH	Reaction of the Culture Medium	Dilution of the Chaulmoogric Acids Totally Inhibiting Growth*
5	1.1	+1.65	—1:20,000
6	1.8	+3.45	1:130,000
7	1.8	+1.7	+1:100,000
8	2.05	+1.65	1:90,000
9	2.05	+1.65	1:130,000
10	2.5	+2.0	1:40,000
11	3.0	+2.0	1:50,000
12	3.6	+1.65	+1:50,000
13	3.8	+1.7	—1:50,000
14	1.8	+1.7	1:100,000
	1.8	0	—1:70,000
15	3.8	+3.45	+1:50,000
	3.8	+1.65	—1:50,000
	3.8	0	+1:25,000
16	4.1	+1.7	1:30,000
	4.1	0	—1:30,000

* In this column the minus sign indicates lower and the plus sign higher than the figures.

From table 2 it is apparent, first, that there is some fluctuation in the antiseptic action with the same titer of the chaulmoogric acids and the same reaction of the culture medium. This fluctuation is due in part to the beforementioned tendency of the chaulmoogrates to skip antiseptic action and is probably wholly explainable on the same grounds, which will be discussed later. But apart from these fluctuations there are more marked and constant variations in antiseptic action which plainly correspond to the difference in titer of the chaulmoogric acids and in the reaction of the culture medium. These variations would have shown up in greater contrast had it not been that some of the lower, higher and intermediate ranges of antiseptic action were not determined, owing to contaminations of the cultures or to the fact that lower or higher dilutions of the chaulmoogric acids were not included in the tests. It appears that a titer of 1.8-2.05 c c of N/1 NaOH per c c of the total fatty acids of chaulmoogra oil gives the maximum antiseptic activity against B. leprae muris, and other experiments have proved that this is true for other acid-fast bacilli. This is the titer obtained by titration in water. Any considerable increase or decrease in the titer from 2.0 markedly depresses the antiseptic and bactericidal activity of the chaulmoogric acids. These experiments also show that a reduction of the acidity of the culture medium to zero likewise depresses the antiseptic activity of the chaulmoogrates. This is brought out specially in Exper. 14, 15, and 16 (table 2), which are strictly comparative tests in the same lot of culture medium with the reaction modified.

This most active titer of 2.0 does not correspond to the titer required to convert all of the chaulmoogric acids into normal sodium salts, as indicated by their titration in alcohol with phenolphthalein as indicator, which requires

a titer of 3.6-3.8. The hydrogen-ion concentration of 1% solutions of the chaulmoogrates at different titers, which were made by the colorimetric method of Clark and Lubs,[40] show the same divergence between the titer of 2.0 and the neutral point as does the titration method, but throws no light on the cause of the greater antiseptic and bactericidal activity of solutions of the lower titer.

TABLE 3

THE HYDROGEN-ION CONCENTRATION OF 1% SOLUTIONS OF SODIUM CHAULMOOGRATES AT DIFFERENT TITERS OF N/1 NaOH

Titer of Solution	Hydrogen-Ion Concentration with Indicators			
	Methyl Red (Range 4.4-6.0)	Brom Cresol Purple (Range 5.2-6.8)	Brom Thymol Blue (Range 6.6-7.6)	Phenol Red (Range 6.8-8.4)
2.0	5.6	5.4	...	...
3.0	...	6.4	6.4	...
3.8	...	...	7.6	7.7

From the tests in table 3 it appears that the solutions of a titer of 2.0 have a hydrogen-ion concentration well on the acid side, while the titer of 3.8 is slightly on the alkaline side.

A possible explanation of the greater antiseptic and bactericidal activity of the neutral solutions with a titer of 2.0 in water over the alkaline solutions with a titer of 3.8 in alcohol is supplied by Power and Gornall.[38] These authors found that in strongly alkaline alcoholic solutions of chaulmoogric acid the normal potassium salt ($C_{18}H_{31}O_2K$) was formed, while in neutral aqueous solutions the acid potassium salt ($C_{18}H_{31}O_2K - 2C_{18}H_{32}O_2$) was formed. Presumably the same reactions would hold true for the sodium salts, in which case the greater bactericidal activity of the neutral, water titrated solution would be due to the acid sodium salts of the chaulmoogric acids.

Having determined the titer of the total fatty acids of chaulmoogra oil that gave the highest antiseptic activity against B. leprae muris, we proceeded to test its antiseptic action on other acid-fast bacilli in cultures. In the case of the several varieties of B. tuberculosis certain technical difficulties were encountered. While our cultures of other acid-fast bacilli will grow in glycerol veal broth in intimate contact with the antiseptic solution, the human and bovine varieties of B. tuberculosis must be inoculated and grow only on the surface of the culture fluid as a waxy membrane. Owing to this growth requirement of tubercle bacilli and to the fatty nature of the bacilli, only the lower surface of the inoculated fragment of membrane comes in contact with and is made wet by the culture fluid and contained antiseptic. The chaulmoogrates are not freely soluble and gradually crystallize out of solution; it is remarkable that this tendency to crystallize out appears to be more marked in weaker solutions. Consequently, in such slow growing cultures as the tubercle bacillus, precipitation of the feebly soluble chaulmoogrates, and possibly also fixation by the contiguous layers of tubercle bacilli, will sufficiently reduce the concentration of the chaulmoogrates in the culture medium to enable the upper layers of tubercle bacilli in the inoculated fragment of membrane that have not been in contact with the antiseptic, to multiply. We have been able to overcome this difficulty in the case of the avian variety of tubercle bacilli which, unlike the human and bovine varieties, can be induced to grow at the bottom of the flask in intimate contact with the antiseptic solution.

In table 4 are collected the results of antiseptic tests of the sodium salts of the total fatty acids of chaulmoogra oil on different acid-fast bacilli, including comparative tests in surface and submerged cultures of the bacillus of avian tuberculosis. In order that the degree of antiseptic activity of the chaulmoogrates may be appreciated, we have included in another column of this table the antiseptic action of phenol tested under the same conditions on certain of these acid-fast bacilli. The figures are based in every case on the results of repeated tests.

TABLE 4

THE ANTISEPTIC ACTION OF SODIUM CHAULMOOGRATES AND OF PHENOL ON CERTAIN ACID-FAST BACILLI

Bacilli	Limits of Complete Antiseptic Action		
	Sodium Chaulmoogrates		Phenol
	In Cultures on the Surface of the Broth	In Cultures Submerged in the Broth	In Cultures Submerged in the Broth
B. leprae muris (Hollmann)	....	1:80,000 to 1:130,000	1:1,000
B. leprae hominis (Levy)	....	1:60,000 to 1:130,000	
B. smegmatis	....	1:80,000 to 1:110,000	1:1,000
B. lymphangitidis bovis	....	1:90,000 to 1:130,000	
B. tuberculosis avis	1:10,000	1:90,000 to +1:140,000	1:1,000
B. tuberculosis bovis	1:10,000 to 1:20,000	........*	
B. tuberculosis hominis	1:10,000 to 1:20,000	........*	

* Will not grow submerged in the broth.

These results show that the sodium salts of the total chaulmoogric acid have a high antiseptic action on all of the acid-fast bacilli tested, but that this antiseptic attains its full activity only when the organisms are growing in complete contact with it in submerged cultures. Comparison of the antiseptic action of the chaulmoogrates on the surface cultures of the human and bovine varieties with that on the surface cultures of the avian variety of B. tuberculosis, and of the surface cultures with the submerged cultures of the avian variety, apparently justifies the conclusion that the chaulmoogrates would have an equally high antiseptic action against the human and bovine varieties if they could be cultivated submerged in and in intimate contact with the antiseptic. Comparison of the chaulmoogrates with the standard antiseptic and bactericide, phenol, brings out in a striking manner the remarkably high antiseptic activity of the former against acid-fast bacilli.

Our experiments with the potassium salts of the total fatty acids of chaulmoogra oil have shown that their antiseptic activity is no greater than, and probably slightly inferior to, the sodium salts. The other ordinary salts of the chaulmoogric acids are insoluble in water and are consequently unsuited for experiments in vitro.

Having established the high antiseptic activity of the sodium chaulmoogrates against acid-fast bacilli, we attempted to determine whether this antiseptic action was merely an inhibition of growth or whether the bacilli were actually killed. We already had, it is true, some evidence that the chaulmoogrates are actually bactericidal and not merely inhibitory of growth. In our preliminary experiments in table 1 transplants from cultures containing chaulmoogrates up to a dilution of 1:100,000 (with the exception of 1:70,000) failed to grow. However, more accurate data on this subject can be obtained

by adding the dilutions of chaulmoogratés to the tubes or flasks of culture medium after inoculation and incubation of the cultures until a definite growth has developed, and then transplanting from the treated cultures to fresh medium at definite intervals. The results on the bactericidal action of the sodium chaulmoogrates on B. leprae muris are given in table 5.

In this experiment the sodium chaulmoogrates killed the bacillus of rat leprosy in 24 hours up to a dilution of 1:75,000 but not at a dilution of 1:100,000 of the chaulmoogric acids. These results do not vary greatly from those obtained in connection with the antiseptic tests recorded in table 1, which gave a bactericidal action up to the dilution of 1:100,000. Probably the limits of complete bactericidal action in vitro of the chaulmoogric acids on this bacillus lie somewhere between 1:75,000 and 1:100,000. The experiment in table 5 also shows that action of the chaulmoogrates for a period of time longer than 24 hours does not increase its bactericidal range. No attempt was made to determine the bactericidal activity of the chaulmoogrates acting for a shorter time than 24 hours because there are reasons for believing that the bactericidal action of the chaulmoogric acids is biologic rather than directly chemical and is consequently slow in action.

An attempt was made to obtain data on the bactericidal action of the chaulmoogrates on B. tuberculosis hominis by the combined in vitro in vivo method. This consisted in subcutaneous inoculations into a series of guinea-pigs of 0.5 c c of saline suspensions of the tubercle bacilli which had been subjected to the action of definite dilutions of the chaulmoogrates for 24 hours at 37.5 C., together with controls inoculated with the same amount of untreated saline suspensions of the bacillus. The results of this experiment as determined by necropsies of the animals are given in table 6.

Some of the guinea-pigs in table 6 died from unknown cause too soon after inoculation to show tuberculous lesions, but these deaths occurred by chance at points in the series at which they do not interfere with the interpretation of the results. This experiment shows a complete bactericidal action of the sodium salts of the total fatty acids of chaulmoogra oil on B. tuberculosis hominis under the conditions of the experiments, only up to a dilution of 1:20,000. There is, however, a probable source of error in the conditions of the experiment that should be pointed out. There are reasons, which will be discussed more in detail later, for believing that the bactericidal action of the chaulmoogrates on acid-fast bacilli is not directly chemical by corrosive or fixative action on their protoplasm, as are salts of the heavy metals; but that its action is indirect or biologic, and is dependent on vital activities of the multiplying bacilli, which attach the chaulmoogric acids to themselves for the purpose of synthesizing their fatty envelopes, and that only when this assimilation has occurred can these peculiar fatty acids exercise the toxic action on the bacilli. Tubercle bacilli suspended in salt solution have their vital activities reduced to a minimum, and would consequently be capable of fixing but little of the chaulmoogrates; the bactericidal action would therefore be greatly reduced.

2. THE ACTIVE BACTERICIDAL PRINCIPLE OF CHAULMOOGRA OIL

Crude chaulmoogra oil has usually been recommended as more effective than the refined product in the treatment of leprosy. If this be true, and since the oil is expressed from the seeds, it might be that small amounts of nitrogenous or other nonfatty substances, such as the glucosid, gynocardin, would be expressed with the oil, and constitute the bactericidal and therapeutic active principle of chaulmoogra oil. Brill and Williams[23] found, on analysis of

TABLE 5

THE BACTERICIDAL ACTION OF THE SODIUM SALTS OF THE TOTAL FATTY ACIDS OF CHAULMOOGRA OIL ON B. LPRAE MURIS

Dilution of Chaulmoogric Acids Added to 24-Hour Cultures	Growth in Cultures Transplanted at Intervals of		
	24 Hours	72 Hours	144 Hours
1:1,000 to 1:75,000	0	0	0
1:100,000	+	+	+
1:125,000	+	+	+
Control	+	+	+

TABLE 6

THE BACTERICIDAL ACTION OF THE SODIUM SALTS OF THE TOTAL FATTY ACIDS OF CHAULMOOGRA OIL ON B. TUBERCULOSIS-HOMINIS BY THE IN VITRO IN VIVO METHOD

Guinea-Pig	Inoculated with Tubercle Bacilli Incubated 24 Hours with Chaulmoogrates of a Dilution of	Fate of Animal	Tuberculous Lesions Found at Necropsy in				
			Point of Inoculation	Glands	Lungs	Liver	Spleen
12a	1:10,000	Killed 139th day	0	+*	0	0	0
12b	1:20,000	Killed 139th day	0	+*	0	0	0
12c	1:30,000	Killed 139th day	+	+	+	+	+
12d	1:40,000	Killed 139th day	+	++	++	+	+
12e	1:50,000	Died 10th day	—	—	—	—	—
12f	1:60,000	Died 132d day	0	++	+++	+	+
12g	1:70,000	Died 102d day	+	+	0	+	0
12h	1:80,000	Died 7th day	—	—	—	+ (?)	—
12i	1:90,000	Died 10th day	+ (?)	—	—	—	—
12j	1:100,000	Died 137th day	0	+	++	++	++
12k	Control	Died 14th day	+	+	+	0	0
12l	Control	Killed 139th day	+	++	++	++	+

* Inguinal gland proximal to inoculation slightly enlarged and caseous, due to the action of the dead tubercle bacilli.

TABLE 7

THE ANTISEPTIC ACTIVITY OF THE SODIUM SALTS OF CHAULMOOGRA OIL AND OF ITS FRACTIONS ON B. LEPRAE MURIS

Chaulmoogra Oil and Fractions	Growth of Bacilli leprae muris in Dilution of														
	1:10,000	1:20,000	1:30,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:90,000	1:100,000	1:110,000	1:120,000	1:130,000	1:140,000	Control
Whole oil	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Total fatty acids	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+
Rogers' fraction A	0	+	0	+	0	+	+	+	+	+	+	+	+	+	+
Rogers' fraction B	0	0	0	+	0	+	+	+	+	+	+	+	+	+	+
Rogers' fraction C	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+
Rogers' fraction B + C	0	0	0	0	0	0	±	0	+	+	+	+	+	+	+
Pure chaulmoogric acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pure hydnocarpic acid	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Residue from the chaulmoogric and hydnocarpic acids	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+

eight samples of chaulmoogra oil, a small amount of nitrogen present which would correspond to from 0.113 to 0.568% of gynocardin. However, our antiseptic and bactericidal tests and the therapeutic experience of Rogers and others indicate that the active principle resides in the fatty acid fraction of the oil; for the method of separation of the fatty acids and their purification by ether would exclude all but the smallest traces of other substances. In order to confirm this and to identify, if possible, the particular fatty acids that are active, the total fatty acids, the several fractions used by Rogers in the treatment of leprosy, and the individual fatty acids were separated by appropriate chemical methods; the whole oil was saponified, and the several fractions and pure fatty acids were converted into sodium salts and their antiseptic activity tested on acid-fast bacilli. The results of these comparative tests on B. leprae muris are given in table 7.

The data in table 7 do not give us the clean cut identification of the active substance of chaulmoogra oil expected from these comparative tests. From the uninterpreted results of these tests it would appear that the total fatty acids are more active than any of its fractions or constituent fatty acids. These apparently paradoxic results are due to certain physical properties of the fractions of higher melting points and of the pure fatty acids which modify the antiseptic and bactericidal activity of these fractions in solutions. Four factors must be considered for the proper interpretation of these data: (1) a bactericidally active fraction; (2) presumably one or more inactive fractions that dilute the active fraction; (3) the low solubility of the salts of fractions having high melting points (Rogers' fractions A and B) and of pure chaulmoogric and hydnocarpic acids, and their consequent tendency to precipitate out of solution; and (4) the well-known physical facts that in mixtures of fatty acids of different melting points, the melting points of the constituent fatty acids are lowered and the solubility of their salts increased.

With these complicating factors in mind the interpretation of the data in table 7 is less difficult. First, it is evident that the active bactericidal principle of chaulmoogra oil is contained in the fatty acid fraction because of the greatly superior activity of the total fatty acids over the whole oil. Second, the progressive increase in activity of Rogers' fractions A, B and C is due to a corresponding progressive decrease in the melting point and increase in solubility of their salts. The increase in the activity of fractions A to C is probably less than it would be if the activity of fractions B and C were not in part neutralized by the increasing proportion of the inactive fraction. Third, fractions B + C are slightly more active than either fraction B or C separately, because the mixture depresses the melting point and increases the solubility of the salts of fraction B which contains a larger proportion of the active fatty acids. Fourth, the last three fractions in table 7 contain together the total fatty acids of chaulmoogra oil which, as we have seen, must contain the active bactericidal substance; yet the activity of each of the three fractions by itself is greatly inferior to that of the total fatty acids. The residue consists chiefly of palmitic acid with some chaulmoogric and hydnocarpic acids or lower isomers that could not be crystallized out of solution. This palmitic acid or other unidentified constituent of the residue cannot be the active substance for the mixture is fluid and its salts freely soluble and, moreover, about 90% of the chaulmoogric and hydnocarpic acids, assumed for the purpose of our argument to be inactive, have been removed, therefore the remaining 10% of theoretical active substance, although not strictly pure, should have an antiseptic activity much greater than that of the total fatty acids. On the contrary,

our tests show that its activity is feeble. The slight antiseptic activity the residue possesses is undoubtedly due to the small amounts of chaulmoogric and hydnocarpic acids contained in it. By this process of elimination we are forced to conclude that the bactericidal activity of chaulmoogra oil is a function of the chaulmoogric acid series, chaulmoogric acid and its isomer, hydnocarpic acid, which are of unique chemical structure among fatty acids, and which constitute about 90% of the total fatty acids of chaulmoogra oil. The feeble antiseptic and bactericidal activity displayed by salts of the pure acids is due to the relatively high melting points and low solubility of their salts, which tend to precipitate out of weak solutions before their bactericidal action becomes effective. When they, together with the small palmitic fraction, are mixed, the melting point of the mixture is depressed and their salts rendered sufficiently soluble to permit the chaulmoogric acid series to exert the high antiseptic and bactericidal activity characteristic of the total fatty acids of chaulmoogra oil.

3. SPECIFICITY OF THE BACTERICIDAL ACTION OF THE CHAULMOOGRIC ACIDS AGAINST ACID-FAST ORGANISMS

Our experiments have shown that the cyclic fatty acids of chaulmoogra oil have a high antiseptic and bactericidal activity against acid-fast bacilli. Is this action specific for acid-fast organisms, or is it general against all bacteria? In order to determine this important point experiments have been conducted with two groups of organisms, the streptothrices and nonacid-fast bacteria. The streptothrices are a group of branching, filamentous fungi, which may by fragmentation develop bacillary forms, and some of them are more or less acid fast. These organisms are regarded by recent investigators as phylogenetically related to the acid-fast bacilli which also, under certain conditions, develop branching forms. Because of this supposed relationship and the variable acid resistance of the different species, the antiseptic and bactericidal action of the chaulmoogrates on this group of organisms is of interest. Table 8 gives the antiseptic action of the sodium salts of the total fatty acids of chaulmoogra oil on different species of streptothrix, together with their morphologic characters and acid-resisting property.

It appears from the results in table 8 that the chaulmoogric acid series have some antiseptic action against streptothrices which is, however, less than against the acid-fast bacilli; that this antiseptic action varies for different species; and that in general the antiseptic action is greater against the bacillary and more or less acid-fast species than against the filamentous and nonacid-fast species.

More interesting and important are the tests of the antiseptic action of the chaulmoogric series on nonacid-fast bacteria (table 9).

These experiments show that the sodium chaulmoogrates are antiseptically and consequently bactericidally inert against nonacid-fast bacteria in dilutions as low as 1:1,000. At such a dilution the growth is usually as luxuriant as in the controls. Dilutions lower than 1:1,000 were not tested, since at this dilution the contrast between the inactivity against nonacid-fast bacteria and the activity against acid-fast bacteria is sufficiently well marked to prove the specificity of the bactericidal activity of the chaulmoogric acids for the latter group of bacteria.

Rogers[1, 36] has stated that therapeutic activity in leprosy and tuberculosis is not peculiar to the fatty acids of chaulmoogra oil, but is common to the unsaturated fatty acids of cod-liver, and presumably other oils. He suggests that the unsaturated fatty acids act on acid-fast bacilli, the coating of which

TABLE 8

THE ANTISEPTIC ACTION OF SODIUM CHAULMOOGRATES ON STREPTOTHRIX

Streptothrix	Morphology	Acid Resistance	Growth in Chaulmoograts at Dilutions of										
			1:5,000	1:10,000	1:20,000	1:30,000	1:40,000	1:50,000	1:60,000	1:70,000	1:80,000	1:100,000	Control
S. eppingeri......	Rods and short filaments	Partial	0	0	0	0	0	0	0	0	+	+	+
S. caprae........	Rods and short filaments	Partial	0	0	0	0	+	+	+	+	+	+	+
S. norcardi......	Rods and short filaments	Partial	0	0	0	+	+	+	+	+	+	+	+
S. hominis.......	Long branching filaments	Vegetative	0	0	+	+	+	+	+	+	+	+	+
S. asteroides.....	Long branching filaments	Vegetative	0	0	+	+	+	+	+	+	+	+	+
S. albus..........	Long branching filaments	Vegetative	0	0	+	+	+	+	+	+	+	+	+
S. bovis..........	Long branching filaments	Vegetative	0	+	+	+	+	+	+	+	+	+	+
S. madurae......	Long branching filaments	Vegetative	0	+	+	+	+	+	+	+	+	+	+

TABLE 9

THE ANTISEPTIC ACTION OF SODIUM CHAULMOOGRATES ON NONACID-FAST BACTERIA

Bacterium	Growth in Sodium Chaulmoogrates: Dilutions from 1:1,000 to 1:100,000	Control
B. coli..	+	+
B. typhosus..	+	+
B. dysenteriae (Shiga)...................................	+	+
B. mucosus...	+	+
B. pestis..	+	+
S. cholerae..	+	+
Staphy. aureus...	+	+
Strep. sp. (non-haemolytic)..............................	+	+

TABLE 10

COMPARISON OF THE ANTISEPTIC ACTIVITIES OF SODIUM CHAULMOOGRATES, SODIUM LINOLEATES AND SODIUM MORRHUATES ON ACID-FAST BACILLI

Bacilli	Dilutions Having Complete Antiseptic Action		
	Sodium Chaulmoogrates	Sodium Linoleates	Sodium Morrhuates
B. leprae muris................	1:80,000 to 1:130,000	—1:1,000 to 1:3,000	1:5,000 to 1:8,000
B. leprae hominis..............	1:60,000 to 1:130,000	1:1,000	1:7,000 to 1:9,000
B. smegmatis..................	1:80.000 to 1:110.000	1:1.000 to 1:4,000	1:8 000
B. lymphangitidis bovis.......	1:90,000 to 1:130,000	1:4,000	1:3,000
B. tuberculosis avis...........	1:90,000 to +1:140,000	—1:5,000 to 1:9,000	1:3,000 to 1:9,000

has been shown to contain unsaturated fatty acids. It is consequently of interest to compare the antiseptic and bactericidal activity of the fatty acids of cod-liver and other oils with those of chaulmoogra oil.

Linoleic acid, the principal fatty acid of linseed and certain other vegetable oils, has the same empiric formula ($C_{18}H_{32}O_2$) as has chaulmoogric acid, and differs from it only in the arrangement of the atoms in its molecule; the molecule of chaulmoogric acid has a carbon ring structure, while the molecule of linoleic acid has its atoms arranged in an open chain. Since the two fatty acids are the chief constituents of the respective oils, and since the salts of the total fatty acids of chaulmoogra oil have been proved to be more highly antiseptic and bactericidal in vitro against acid-fast bacilli than any of its fractions, it has been considered fair to use the sodium salts of the total fatty acids of both oils for comparative tests.

Rogers' sodium morrhuate consists of the sodium salts of the total fatty acids of cod-liver oil. Our knowledge of the chemistry of cod-liver oil is far from exact; but its composition, as is to be expected of an animal oil extracted from an organ having such metabolic activities as the liver, is very complex. A considerable number of fatty acids, including oleic, palmitic, steric, myristeric, palmitoleic, gadoleic, erucic and therapic acids, together with two alkaloids, asselin and morrhuin, traces of iodin and sometimes bromin, and butylamin, amylamin, herylamin and hydrodimethyl-pyridin, have been reported as occurring in cod-liver oil. However, all but traces of substances other than the fatty acids would be excluded by the method of preparation of Rogers' sodium morrhuate. So far as is known, cod-liver oil does not contain fatty acids of the chaulmoogric series nor any fatty acids having a cyclic structure.

In table 10 are collected the results of antiseptic tests of sodium linoleates and sodium morrhuates compared with sodium chaulmoograte on various acid-fast bacilli. In this table, as in the preceding tables, where two figures are given they represent the lowest and highest range of antiseptic action obtained in repeated experiments.

These experiments show that the sodium linoleates and morrhuates have a slight antiseptic action on acid-fast bacilli. This is probably a nonspecific soap action—for the sodium salts of the fatty acids are soaps, of which dilutions up to 1:5,000 give decidedly soapy and up to 1:10,000 perceptibly soapy solutions—in which the fatty capsules of acid-fast bacilli are injured (emulsified) by the more concentrated solutions of the soaps. In consequence of this low antiseptic activity, and since bactericidal action is never greater, and is usually less, than antiseptic action, it has not been considered necessary to test the bactericidal actions of the linoleates and morrhuates on acid-fast bacilli. In strong contrast to this relatively feeble soap action of the linoleates and morrhuates stands the high antiseptic and bactericidal activities of the chaulmoogrates against acid-fast bacilli, activities which these comparative experiments indicate are specific to the cyclic fatty acids of the chaulmoogric series.

DISCUSSION

It is convenient in discussing the experimental data to follow the outline given in the introductory paragraphs, and to determine how much information we have obtained by these experiments in vitro bearing on the several problems involved in the fatty acid therapy of leprosy and tuberculosis.

The first problem was the method of therapeutic action of chaulmoogra oil in leprosy. This problem presented three chief possibilities: (1) that the reputed therapeutic effect is due to direct bactericidal action of chaulmoogra oil or some of its constituents on B. leprae; (2) that chaulmoogra oil acts indirectly by stimulating the tissues to react against the invading organisms, and (3) that chaulmoogra oil is inactive and improvement of patients following its use is spontaneous. The first of these possibilities, that of direct bactericidal action, was the simplest and most attractive one, and would place chaulmoogra oil or its active constituent among the true chemotherapeutic agents. For this reason the antiseptic and bactericidal activities of chaulmoogra oil and its constituents were first investigated. Our experiments have shown that the sodium salts of the total fatty acids of chaulmoogra oil have a very high antiseptic and bactericidal activity against acidfast bacilli. This bactericidal action extends to a dilution of about 1:100,000, and the antiseptic action is perceptible to a dilution of at least 1:1,000,000. In the light of these results and of the facts that these high antiseptic and bactericidal activities have proved to be peculiar to certain fatty acids of chaulmoogra oil and specific against acid-fast bacilli, as will be discussed, we have not considered it necessary or profitable to investigate hypothetical indirect action of chaulmoogra oil in leprosy; and we believe that it can be concluded with reasonable certainty that any therapeutic action which chaulmoogra oil may have in leprosy is due to its direct antiseptic and bactericidal action on B. leprae.

In this connection the question of the relation of bactericidal dilution in vitro to the therapeutic dosage in vivo of the chaulmoogratcs naturally arises. If, as is true, the limits of complete bactericidal action in vitro is the dilution of about 1:100,000, but the therapeutic dosage intravenous in leprosy is in the proportion of from 1:2,000,000 to 1:500,000 of the body weight, how can we account for the therapeutic effect claimed in leprosy on the basis of even the high bactericidal action of the chaulmoogrates? Our experiments in vitro have, for the purpose of analysis, intentionally excluded the factors that may be supplied by the host in the action of the chaulmoogrates on acid-fast bacilli in vivo. There are three such factors which, individually or conjointly, might account for the apparent discrepancy between the bactericidally active dilution in vitro and the therapeutically active dosage in vivo. First, other chemotherapeutic agents are known, such as arsphenamin, which act therapeutically in the animal

body at higher dilutions than they do germicidally in vitro. This intensified action in vivo is probably due to some chemical modification of the substance brought about by the tissues of the host. Second, fats and fatty acids are not, like some drugs, rapidly broken down and excreted by the animal body, but are stored in the tissues for metabolic use. Consequently, in the regular and long continued administration of the chaulmoogric acids in the treatment of leprosy there would be an accumulation of the chaulmoogric acids or their esters in the body that might well reach the concentration of bactericidal action. This might explain in part the slow action of the chaulmoogrates in leprosy. Third, the high antiseptic range of the chaulmoogrates, which is perceptible up to a dilution of at least 1 : 1,000,000, above the complete bactericidal activity may be an important factor in the therapeutic results. Such a high antiseptic action, although incomplete, might in conjunction with the tissue reactions be sufficient to restrain the multiplication of the bacilli; or the inhibitory action of the chaulmoogrates might so injure or reduce the vitality of the parasites that the natural resistance of the host would be able to overcome them. The experimental determination of the actual relation between bactericidal dilution in vitro and therapeutic dosage in animals will be considered in a later article.

The second problem was that of the active principle of chaulmoogra oil. Our experiments have shown conclusively that the bactericidally active principle is contained in the fatty acids of this oil. This is in accord with the experience of Rogers,[29, 30] Hollmann and Dean[11] and others that the fatty acid fraction of chaulmoogra oil is most active therapeutically in leprosy. But, whereas Rogers claims superior therapeutic results with first one fraction and then another of the chaulmoogric acids, we have obtained the highest bactericidal activity in vitro with the salts of the total fatty acids. Our results are due to the greater solubility of the salts of the mixed fatty acids, and do not indicate that all of the fatty acids of chaulmoogra oil are bactericidally active. The low solubility of the salts of the individual fatty acids and their consequent tendency to crystallize out of weak solutions have interfered with the direct identification of the specific fatty acids of chaulmoogra oil possessing this bactericidal property; but by indirect methods of exclusion we have been able to satisfy ourselves that the small palmitic acid fraction is inactive, and that the bactericidal activity is a function of the chaulmoogric acid series, chaulmoogric and hydnocarpic acids and possibly lower isomers of this

series, which together constitute about 90% of the fatty acids of chaulmoogra oil. The chaulmoogric acid series, it will be recalled, have been shown by the researches of Power and Gornall,[38] and of Brill[23] to have a peculiar molecular structure containing a closed carbon chain, which is found in no other known fatty acids.

The third problem was the degree of specificity of the bactericidal activity of the chaulmoogric acid series. Bactericides may be either nonspecific, such as phenol and salts of the heavy metals, which act against all bacteria and show no marked variation in their action on different species, except in so far as it may be modified by the development of resistant spores by certain species; or they may be more or less specific and act more strongly against certain species or groups of bacteria, as is the case with ethylhydrocuprein against the pneumococcus group. Our experiments have shown that the chaulmoogric acids belong to the latter class of bactericides, and that they have a very sharply limited group specificity. They possess a high bactericidal activity against all members of the acid-fast group of bacilli and are inactive against all other bacteria tested. This group specificity is probably connected, not with acid-fastness as such nor with any protoplasmic relationship of the different acid-fast organisms, but with the fat metabolism of acid-fast bacilli and the mechanism of the bactericidal action of the chaulmoogric acids. It is known that growths of acid-fast bacilli contain large amounts of fats and waxes (20-37% in case of the tubercle bacilli), which are intimately connected with the bacterial cell in that they are a product of its metabolism, and constitute its protective capsule. Kendall, Walker and Day[41] have shown that acid-fast bacilli produce a soluble lipase in their growth, which is probably concerned in the metabolism of this fatty capsule. Rogers has suggested that the unsaturated fatty acids act on acid-fast bacilli by injuring the protective fatty capsules of acid-fast bacilli. This assumption, he believes, is supported by his observations that the bacilli excreted by patients undergoing treatment show irregular acid-fast staining. Such bacilli, deprived of their protective fatty capsules, would be exposed to the destructive action of the tissues and body fluids. Our experiments in vitro, however, have failed to show the slightest action of the sodium chaulmoogrates, in any concentration and acting for any length of time, on the staining characters or morphology of acid-fast bacilli.

[41] Jour. Infect. Dis., 1914, 15, p. 443.

An hypothesis which seems best to explain the mechanism of the bactericidal action of the chaúlmoogric acids and their specificity for acid-fast bacilli is that these fat elaborating bacilli attempt to utilize the chaulmoogric acids to build up their fatty capsules, and that these cyclic fatty acids contain a group or an arrangement of atoms which is toxic for the bacterial cell. In the terminology of Ehrlich's side chain theory, we may express this reaction by saying that chaulmoogric acid possesses an haptophore group which becomes attached to the receptor or side chain of the acid-fast bacillus, and a toxophore group which, after attachment, exerts a toxic action on the bacillus. On the basis of this hypothesis, the chaulmoogric acids are not bactericidal against nonacid-fast bacteria because these organisms, not elaborating a fatty capsule, do not necessarily use fats in their metabolism, and consequently do not possess the proper receptors for the haptophore group of the chaulmoogric acids.

This hypothesis would also help to explain the irregularities encountered in our bactericidal experiments in vitro, such as skipping and variation in antiseptic and bactericidal action in different tests. It is well known that fat metabolism, as indicated by acid-fastness, is not a constant or even a vitally necessary function of acid-fast organisms, since there are usually a variable number of nonacid-fast individuals in young cultures of acid-fast bacilli, and it has been shown experimentally that acid-fastness can be modified and even wholly suppressed in some species by conditions of growth. In our experimental cultures such nonacid-fast individual bacilli, in which fat metabolism was temporarily dormant, would by our hypothesis be immune to the action of the chaulmoogrates. After the greater part of the chaulmoogrates in the culture had become fixed by the acid-fast organisms or precipitated in virtue of their slight solubility, these nonacid-fast individuals might be able to multiply and resume their fat metabolism and acid-fast property unrestrained.

The fourth and last major problem investigated was that of the presence of bactericidally active fatty acids in other oils. It will be recalled that Rogers has stated that the salts of the unsaturated fatty acids of both chaulmoogra and cod-liver oils, and by implication the unsaturated fatty acids of any oil, are equally efficacious therapeutically in either leprosy or tuberculosis. Our experiments do not support the claim of Rogers. They show that the high specific bactericidal activity against acid-fast bacilli is not a property common to unsaturated fatty acids, but that it is restricted to the cyclic fatty acids

of the chaulmoogric series. The number of fatty acids that we have investigated is limited; but we believe that they have been particularly well chosen. Linoleic acid has an empiric formula ($C_{18}H_{32}O_2$) identical with that of chaulmoogric acid, and differs from the latter only in the arrangement of its atoms in an open chain instead of in a ring. Rogers' sodium morrhuate contains a considerable number of fatty acids, none of which are known to be of cyclic structure, and for which specific therapeutic action in leprosy and tuberculosis is claimed. We have demonstrated that neither of these have any marked bactericidal activity against acid-fast bacilli, but are relatively inert. Therefore we believe the conclusion to be warranted that the specific bactericidal activity of fatty acids against acid-fast bacilli is a function of the carbon ring structure of the molecules of the chaulmoogric acid series, a structure known to exist only in chaulmoogra oil and in oils of certain plants closely related to Taraktogenous kurzii from whence chaulmoogra oil is obtained.

These experiments in vitro supply certain definite information on the major problems of the purposed investigation of the fatty acid therapy of leprosy and tuberculosis, namely: (1) the method of therapeutic action of chaulmoogra oil in leprosy; (2) the active principle of chaulmoogra oil; (3) the specificity of its action on the acid-fast group of bacteria, and (4) the limitation of the active principle to the cyclic fatty acids of chaulmoogra oil. Much of this information could not have been obtained by other methods of experimentation; but the actual chemotherapeutic value of the chaulmoogric acid compounds in the treatment of infections due to the acid-fast group of bacilli, especially tuberculosis, remains to be proved by experiments on animals and by clinical experience. It is strongly recommended, however, that clinical trial of the chaulmoogrates in tuberculosis await the results of the animal experiments now in progress; for the indiscriminate use of this drug may arouse false hopes and be not without danger to the patients.

SUMMARY

Chaulmoogra oil contains bactericidal substances that are about one hundred times more active than phenol.

The bactericidally active substances of chaulmoogra oil are the fatty acids of the chaulmoogric series, chaulmoogric and hydnocarpic acids, and possibly lower isomers of this series.

The bactericidal activity of the chaulmoogric acid series is specific for the acid-fast group of bacteria, and inactive against all other bacteria tested.

This specific bactericidal activity against acid-fast bacteria is a function of the carbon ring structure of the molecule of the chaulmoogric acid series which, so far as known, is found only in chaulmoogra oil and in oils of certain plants closely related to Taraktogenous kurzii.

The fatty acids of cod-liver oil, the salts of which constitute Rogers' sodium morrhuate, used in the specific treatment of tuberculosis, do not possess the specific bactericidal activity of the chaulmoogric acid series.

These facts supply a scientific basis for the use of chaulmoogra oil and its products in leprosy.

Our experiments do not support the claims of Rogers for sodium morrhuate in the specific therapy of tuberculosis.

The bactericidal activity of the chaulmoogric acids against all members of the acid-fast group of bacilli, together with the clinical results obtained from their use in leprosy, furnish theoretical grounds for the application of the chaulmoogrates to the therapy of tuberculosis.

Experiments on animals are now in progress to determine whether or not the chaulmoogric acid series have any practical value in the chemotherapy of tuberculosis.